Denaturation/Renaturation of Organophosphorus Acid Anhydrolase (OPAA) Using Guanidinium Hydrochloride and Urea

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The understanding of how protein unfolds/refolds is a key to the development of any protein/enzyme based detection system. Using organophosphorus acid anhydrolase (OPAA) as the model protein, a guanidinium hydrochloride and urea denaturation/renaturation study was conducted and measured both optically and enzymatically. As expected, the highly autofluorescent tryptophan moiety (Ex. 280/Em. 340nm) decreased in intensity and red-shifted as denaturant concentration was increased and vice versa upon renaturation; thereby indicating conformational changes. Similar results were obtained with circular dichroism as the peak representing the alpha-helix conformation decreased as denaturant concentration was increased. Likewise, OPAA activity decreased with increasing urea concentration. However with guanidinium hydrochloride little to no enzymatic activity upon denaturation or renaturation was detected. With the fundamental understanding of protein chain folding, one can design nanoencapsulated enzyme systems for detection and decontamination of chemical warfare agents.

INTRODUCTION:

Protein folding is a very unique and complex process. In 1969, Levinthal stated that a polypeptide chain would require an astronomical time to explore at random all possible conformations to finally reach the native state.[1] Unfortunately, the development of any protein/enzyme based detection system depends on the fundamental understanding of protein chain folding.

The enzyme, organophosphorus aid anhydrolase (OPAA), is a well-characterized enzyme for its ability to safely decontaminate G-type nerve agents. Written in the Joint Service Science and Technology Chemical and Biological Decontamination Master Plan, the objective of the decontamination technology advancement efforts is to develop systems that are rapid and effective in detoxifying CB agents, environmentally safe, have no impact on the operational effectiveness of the hardware being decontaminated, and minimize the logistical impact on operations.[2] Hence an on-going multiagency effort is developing an Advanced Catalytic Enzyme System (ACES) that will contain enzymes for the detoxification of nerve agents, related pesticides, sulfur mustard, bacterial cells, and anthrax spores.[2] However, the development of such system requires the need to understand the enzyme behavior.

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OPAA was oringinally isolated from *Alteromonas* spJD6.5 by Cheng et al in 1989 and subsequently sequenced and cloned. (Figure 1) The protein was found to be a single polypeptide composed of 517 amino acids with molecular weight range of 58 kDa. It has a 22% amino acid homology with human prolidase.[3,4] Recently, the three dimensional protein crystal structure of the OPAA trimer was determined,[5] and the enzyme will be commercially available in December 2004.[6]

Numerous analytical techniques have been employed to address the protein folding dilemma. Two common techniques are fluorescence spectroscopy and circular dichroism (CD) for the following advantages: experiment can be carried out in solution, only minute amounts of material are required for analysis, and the methods are non-destructive such that the samples can be recovered after the experiments. Proteins naturally contain intrinsic aromatic amino acid chromophores which is much more sensitive to changes in the environment of the chromophore than is light absorption, making it an excellent spectroscopic probe for investigating conformational changes in proteins for it is much more sensitive to changes in the environment of the chromophore than is light absorption, quick, and simple.

This report highlights our preliminary results obtained from the protein unfolding/refolding study of OPAA in the presence/absence of denaturants, i.e., urea and guanidinium hydrochloride.

MATERIALS AND METHODS:

OPAA Samples

All OPAA samples were prepared and purified using protocols established previously.[7,8] Protein concentration was determined using a Coomassie Plus Protein Assay Reagent Kit (Pierce), and the stock concentration was evaluated to be 10 mg/mL. Samples were aliquoted and stored in the freezer until use.

Protein Denaturation/Renaturation Study

1. Preparation.

An 8-M urea stock solution was prepared by dissolving 14.41g of dry urea (Fisher Scientific) into 30 mL of 10 mM Bis-Tris Propane, pH 7. Similarly, 22.93 g of dry guanidinium hydrochloride (Fisher Scientific) were dissolved in 30 mL 10 mM Bis-Tris Propane (10BTP). Subsequent dilutions were prepared. All solutions were set to measure pH 7.2.

2. OPAA Denaturation/Renaturation Procedure.

Both denatured and renatured sample sets were prepared in urea and guanidinium hydrochloride as described in the literature.[6,7] For a final protein concentration of 20

μg/mL, the stock solution used for the denatured sample set was native protein while a pre-denatured protein was used for the renatured sample set. All samples (protein and buffer solutions) were measured for fluorescence after 24 hour incubation at 25 ^{o}C.

3. Fluorescence Measurement.

Samples were transferred into a 1-cm quartz cell and measured using a Fluorolog 2-Tau-2 (Spex Industries) system. All slits were set at 2.5 nm bandwidth, and sample fluorescence was collected at 1 nm increments for 0.1s integration using a xenon arc lamp excitation source. Fluorescence spectra were recorded at 90^{o} from the excitation source at 280 nm. Both Excel (Microsoft) and Grams 32 software packages (Galactic Industries) were employed for data analysis.

4. Circular Dichroism (CD) Measurement

Samples were transferred into a 1-cm quartz cell. A J-810 (Jasco Corp.) was employed for the CD measurements. Scans were collected from 200-300 nm. Data was transferred into Excel for analysis.

4. OPAA Activity Measurements.

Enzyme activity was measured by monitoring the release of fluoride (F^-) ion with an ion specific electrode using the standard procedures as reported previously.[1-4,7-8] The electrode was calibrated at each denaturant concentration prior to measurement using standard fluoride solutions in the 10 mM Bis-Tris Propane pH 7.2 buffer containing 1 mM $MnCl_2$ at 25 ^{o}C. Under identical conditions, the amount of fluoride released was recorded at 2 minute intervals from the enzyme's interaction with diisopropyl fluorophosphonate (DFP) as substrate (Sigma Chemical Company). All readings were corrected for spontaneous DFP hydrolysis under identical conditions. From this corrected value the enzyme activity of OPAA 315 was calculated. One unit of OPAA activity is defined as catalyzing the release of 1.0 μmol of F^- per minute. Specific activity is expressed as units per milligram of the protein.

RESULTS and DISCUSSION:

OPAA Activity Calculation.

Activity measurement is based on the following equation:

$$R = C_{\Delta} - C_{DFP}\left(\frac{E_1}{V_1 xT}\right)$$

whereby: R = enzyme activity in micromoles/min/mL
$C_{\Delta} = C_2 - C_1$
C_{DFP} = DFP spontaneous rate
C = Fluoride ion concentration in μM measured from ISE meter

$E_1 = 1/E$

E = sample volume in mL

$V_1 = 1000/V$

V = Total assay volume in mL = 2.5 mL

T = time of assay in minutes = 4

The relative OPAA activities for both denatured and renatured as a function of urea and guanidinium hydrochloride (GuHCl) are shown in Figure 2. Unlike urea, OPAA exhibited little to no activity in GuHCl. This might be a result of two reasons: (i) GuHCl is a stronger denaturant than urea, and (ii) GuHCl is a salt that may alter the stability of a protein at low denaturant concentration through an ionic strength effect.[9]

At 280 nm excitation, both intrinsic chromophoric amino acids tryptophan and tyrosine are interrogated. The fluorescent profiles of OPAA's denaturation and renaturation at varying urea concentrations are plotted in Figures 3. The intensity of the tryptophan emission at 340 nm decreases and red-shifts as denaturant concentration is increased indicating protein unfolding which corresponds to fluorescence maximum of tryptophan in aqueous solution at 350 nm.[10] Another evidence of protein unfolding is the presence of the tyrosine peak at 303 nm appearing. This effect occurs during unfolding as the distance between the tryptophan and tyrosine residue increases, and the energy transfer becomes less efficient.[11] During the renaturation process, the protein refolds as indicated by the increasing fluorescence peak, blue shift, and disappearance of the 303 nm band. However, the $1x10^6$ difference at 1 M urea between the renatured and denatured profiles indicates either protein loss or improper refolding.

The fluorescent profiles of OPAA's denaturation and renaturation in varying guanidinium hydrochloride concentrations are plotted in Figure 4. Similar to the urea, the intensity of the tryptophan peak at 340 nm decreases and red-shifts as guanidinium hydrochloride concentration increases. Unlike urea, the presence of the 303 nm band is still visible upon renaturation suggesting improper refolding. In addition, the difference between the renatured and denatured OPAA at 1 M is very large, $1.5x10^6$. Guanidinium hydrochloride appears to have a stronger effect on OPAA's renaturation than the urea confirming the enzyme activity result.

The conformational stability of a protein molecule is customarily described by the difference in free energy between the unfolded and native states, ΔG.[12] In the native state, many of the protein functional groups are buried in the core excluded from water. Numerous forces are involved in protein stability: van der Waals interaction, hydrogen bonds, electrostatic interaction, configurational entropy, and water. Within the core, specific interactions and tight packaging essentially fix the amino acid side chains making their motion somewhat restricted. Upon unfolding, the interactions between protein groups within the core are disrupted and replaced with interactions with the solvent. Assuming a two-state mechanism whereby native $\Leftrightarrow$ unfolded, the free energy change (ΔG_u) for the folding/unfolding reactions can be calculated.[13] This model assumes that at equilibrium only the fully folded or native protein and the fully unfolded or denatured protein are present at significant concentrations since the concentration of

intermediates for many proteins at equilibrium is relatively small to the concentrations of the native and denatured proteins.

$$\Delta G = -RT \ln K$$

whereby:
R = gas constant = 1.987 cal/deg/mol
T = absolute temperature = 298 K
K = equilibrium constant = F_u/F_f
F_u or F_f = fractional intensity of folded/unfolded protein

$$\Delta G_u = \Delta G(H_2O) - m[\text{denaturant}]$$

$\Delta G(H_2O)$ is the free energy change for the folding/unfolding reaction at 25^{o}C in the absence of denaturant or the conformational stability. Using these equations, the calculated conformational stability of the OPAA protein in urea and guanidinium hydrochloride were respectively 1.7 kcal/mol (-864 cal/mol/M) and 2.4 kcal/mol (-507 cal/mol/M). The value in the parenthesis is the slope (m), which measures the dependence of free energy on denaturant concentration. This value depends on the amount or composition of the polypeptide chain that is freshly exposed to solvent upon unfolding.

Initial circular dichroism studies were conducted using the 4 and 8 M denaturant solutions and compared to native OPAA. Intensities are reported at 209, 222, and 216 nm regions as shown in Table 1. Results indicate presence of α-helix and possibly β-sheet in the secondary structure; as confirmed by the protein structure shown in Figure 1. As denaturant concentration increases from 4 to 8 M, the intensity of the peak decreases by three times for urea and about two times for GuHCl. Similar effect was observed upon renaturation.

CONCLUSIONS:

OPAA (*sp*JD6.5) is a unique protein with high level enzymatic activity against a wide variety of toxic organophosphorus compounds including chemical G-type nerve agents. In this report, both denaturation and renaturation profiles of OPAA in potent denaturants, urea and guanidinium hydrochloride, are shown. The protein unfolding/refolding characteristics of OPAA have been successfully demonstrated. Preliminary CD studies also show that the secondary protein structure can be deduced. It is envisioned that such information will benefit the ACES development toward the design of a nanoencapsulated enzyme systems for the detection/ decontamination of chemical warfare agents.

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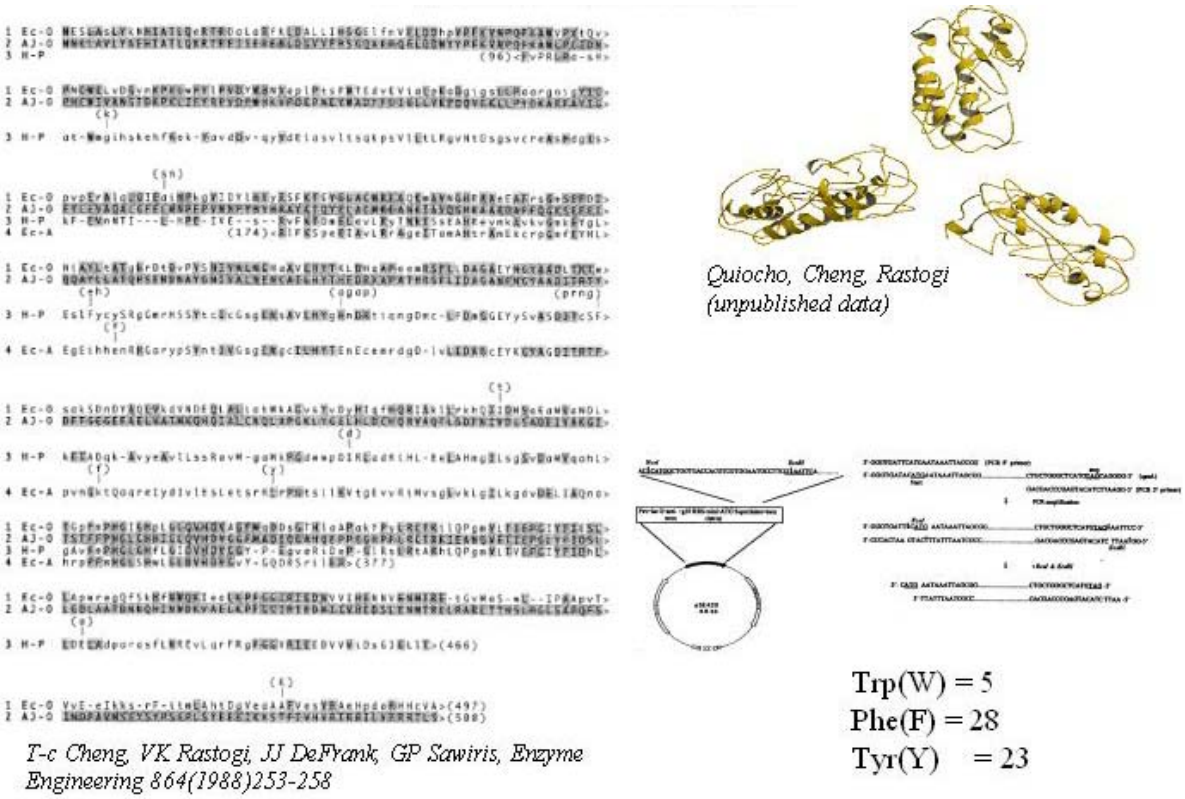

Figure 1. OPAA protein sequence (left), 3-D crystalline protein structure (top right) and gene expression (bottom right).

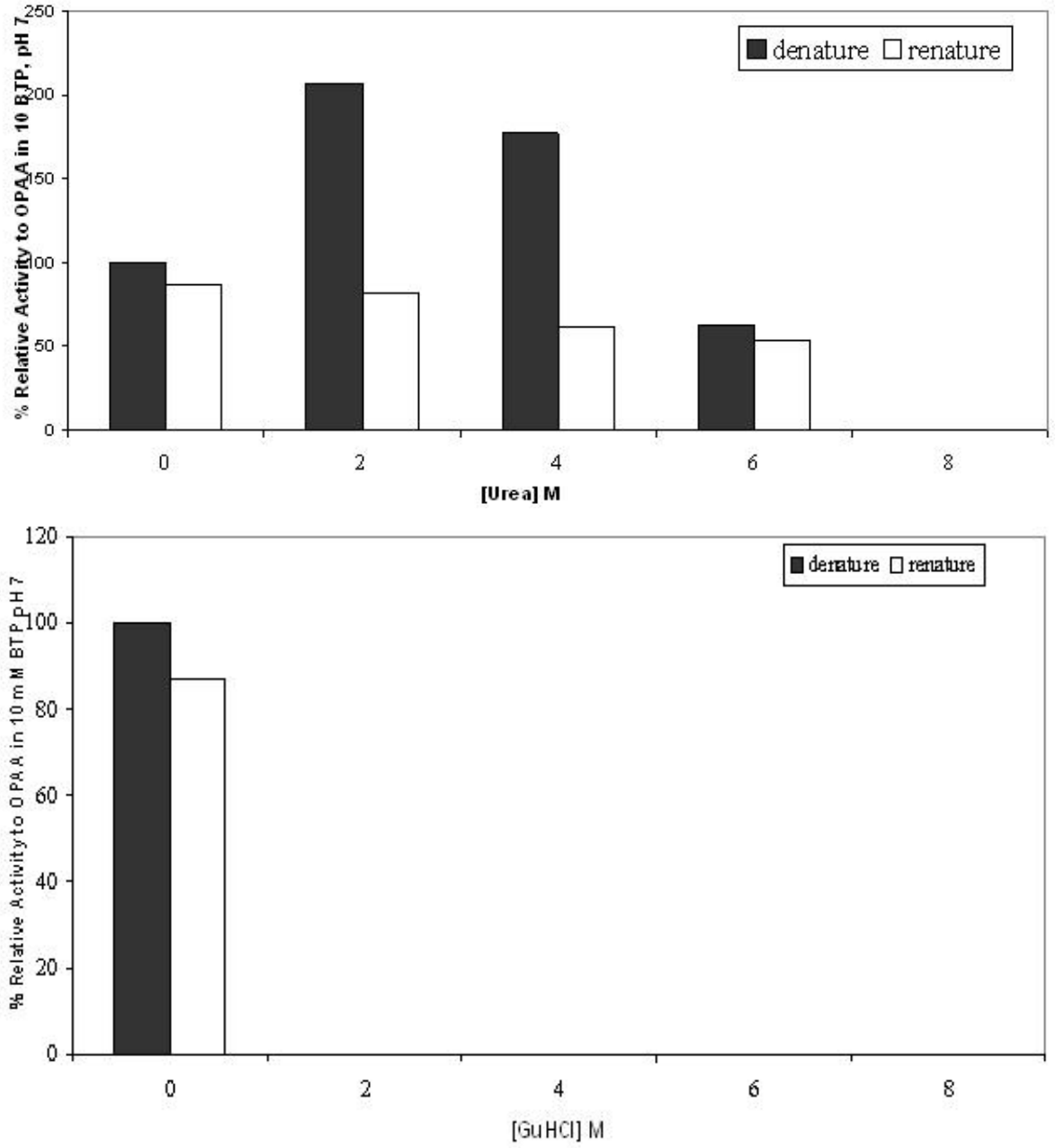

Figure 2. Enzyme Activity Profiles. The denatured bars are shaded.

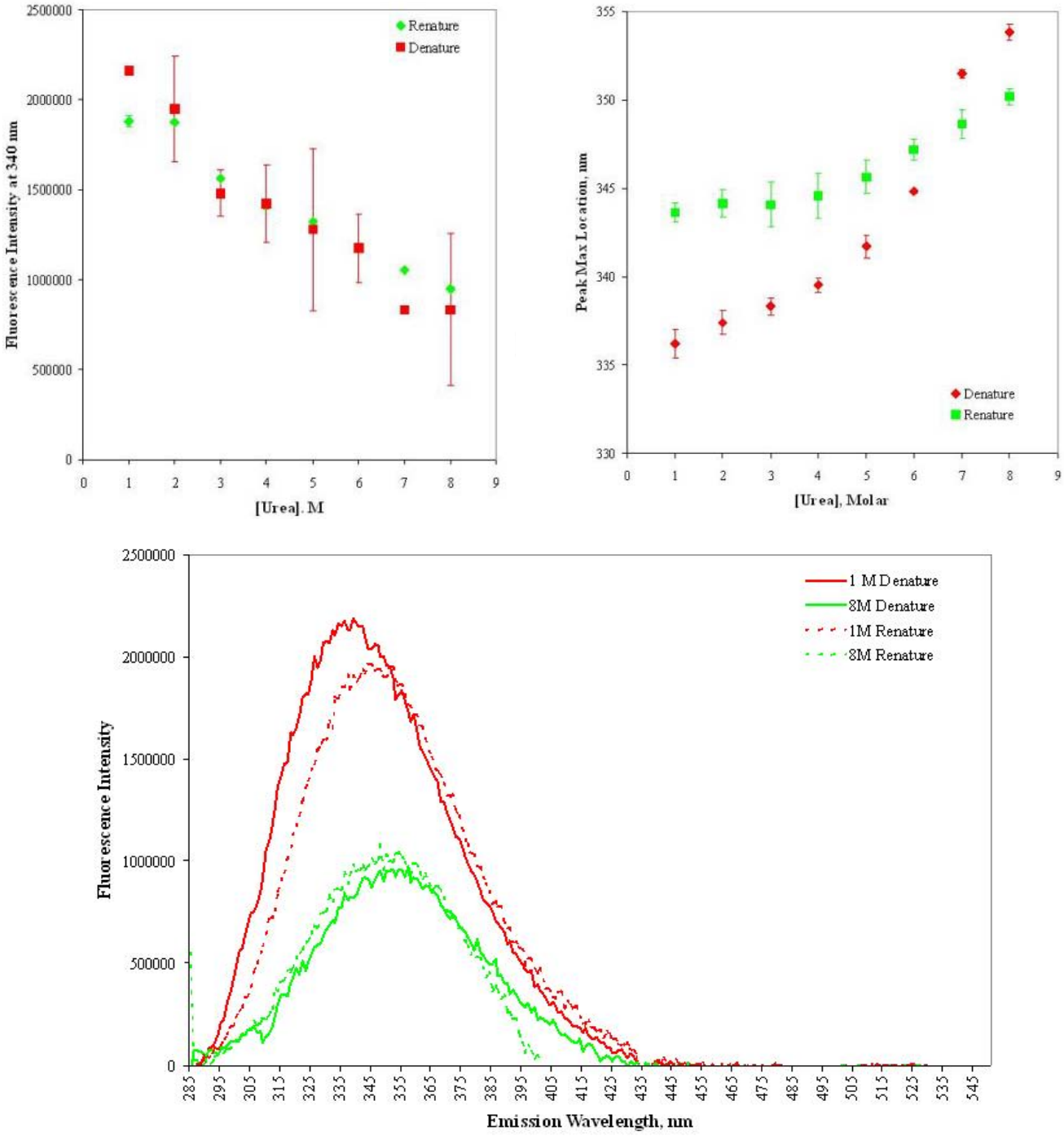

Figure 3. Urea Denaturation/Renaturation Profiles. Shown are plots of fluorescence intensity at 340 nm versus denaturant concentration in mol/L, wavelength of peak maximum versus concentration, and sample fluorescent profile for 1 M and 8 M denatured/renatured samples. In the two top profiles, denatured data are depicted by diamonds, and renatured data are squares. In the fluorescent profile, the renatured samples are dashed lines.

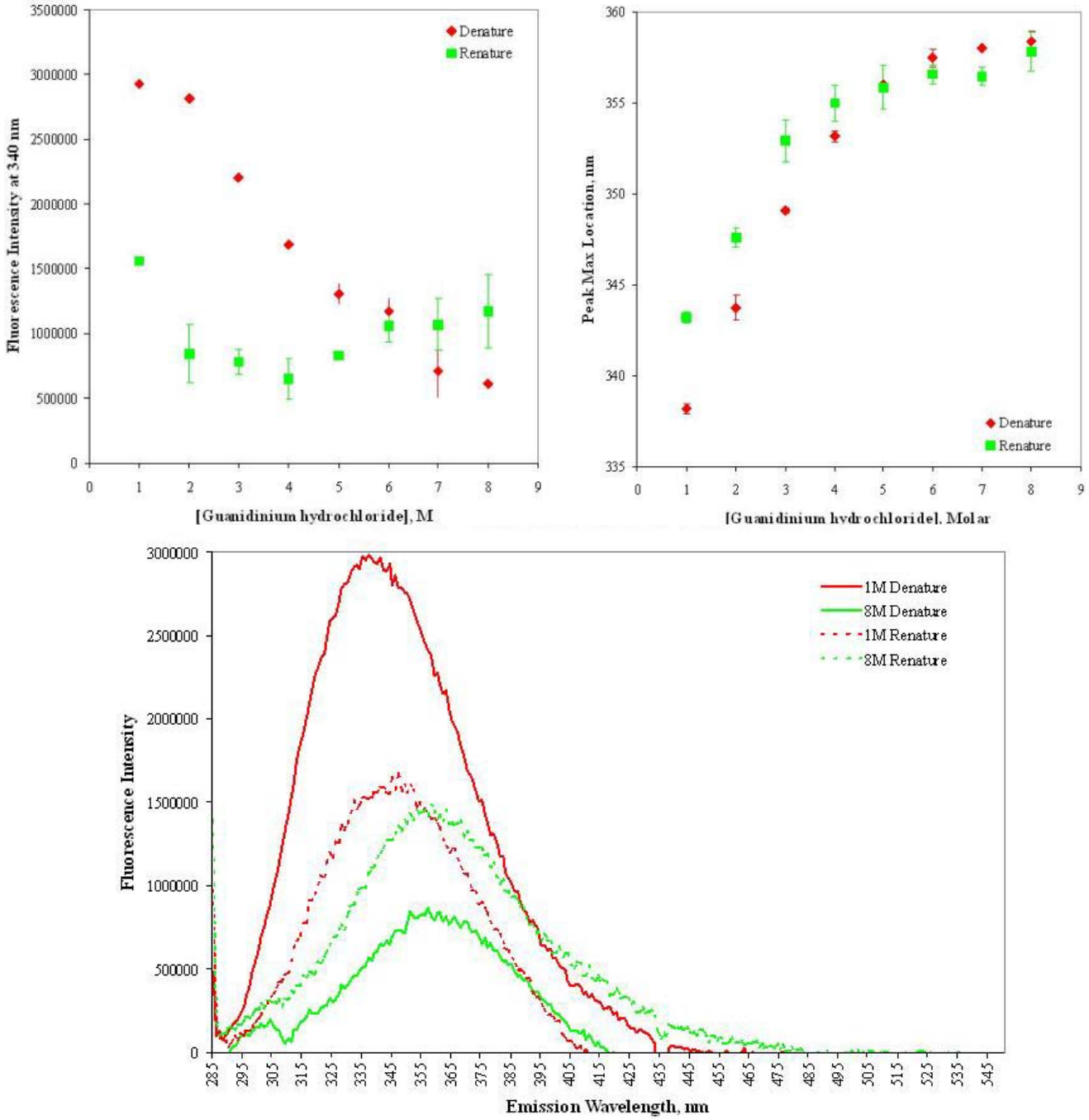

Figure 4. Guanidinium hydrochloride Denaturation/Renaturation Profiles. Shown are plots of fluorescence intensity at 340 nm versus denaturant concentration in mol/L, wavelength of peak maximum versus concentration, and sample fluorescent profile for 1 M and 8 M denatured/renatured samples. In the two top profiles, denatured data are depicted by diamonds, and renatured data are squares. In the fluorescent profile, the renatured samples are dashed lines.

Table 1. Preliminary circular dichroism results using 4 M and 8 M denaturant solutions. Data for native OPAA and buffers are also shown. Intensities are reported at 209, 222, and 216 nm regions.

GuHCl			
	I 216	I 209	I 222
Native OPAA	-14.855	-14.858	-14.581
4 M Denature	-4.6084	-6.4446	-5.0632
8 M Denature	-3.6751	65.296	-3.1981
4 M Renature	-4.1839	2.0194	-3.2405
8 M Renature	-5.0495	-4.7166	-4.7185
8M buffer	-4.6502	33.024	-1.7987
10 BTP	2.3143	3.0043	1.7309

Urea			
	I 216	I 209	I 222
Native OPAA	-14.855	-14.858	-14.581
4 M Denature	-16.091	-62.064	-16.172
8 M Denature	-4.1028	8.496	-6.2764
4 M Renature	-9.2242	-10.194	-7.1107
8 M Renature	-7.5954	-5.3436	-7.202
8M buffer	6.0375	35.072	-2.3926
10 BTP	2.3143	3.0043	1.7309